culture plates (Freundlich and Avdalovic, 1983). Purified monocytes were then differentiated on tissue culture plates for 7-10 days and infected as described (Kornbluth et al., 1989).

Cell Cycle Analyses

DNA content of nuclei was determined as described (Nicoletti et al., 1991) by lysing plasma membranes, staining nuclear DNA with propidium iodide, and quantitating the relative DNA content of nuclei using the Becton-Dickinson FACSORT fluorescence activated cell sorter. The proportion of nuclei in G0/G1, S, and G2/M was determined using the MODFIT DNA analysis software.

In Situ Hybridization

Cells were fixed and hybridized overnight in hybridization buffer (Berthold and Maldarelli, 1996) to a ^{32}P-labeled DNA probe generated by random priming (multiprime DNA labeling kit, Amersham) from a 5.3 kb DNA fragment including the U5-*gag/pol*, *vif*, and a portion of the *vpr* region of HIV-1 (pNL4-3 nucleotides 433-5743). Cells were washed as described (Berthold and Maldarelli), then air dried, subjected to autoradiography, examined by microscopy, and scored for HIV-1 infection when signal was present within the nuclei.

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In Response to Freed et al.

Our recent proposal that the C-terminal tyrosine phosphorylation of MA reveals the karyophilic potential of this protein within the context of the HIV-1 preintegration complex relied on a series of experiments which helped to define the components of this complex and to identify their interactions among each other and with the cell nuclear import machinery, and which examined their intracellular trafficking during the early steps of the viral life cycle (Gallay et al., 1995a, 1995b). Using tyrosine kinase inhibitors, Bukrinskaya et al. (1996) confirmed that MA tyrosine phosphorylation promotes the nuclear targeting of the viral DNA and facilitates HIV-1 infection of nondividing cells, even though they suggested that the C-terminal residue might not be the sole phosphotyrosine present in MA.

Freed et al. previously questioned the role of the MA NLS in HIV-1 infection of macrophages (Freed and Martin, 1994; Freed et al., 1995). In line with this position, they now present data contesting the participation of the C-terminal phosphorylation of MA in this process. To address this controversy, we first compared the status of MA tyrosine phosphorylation of their and our viruses. We found that in both cases the C-terminal residue was the only phosphotyrosine of MA, in agreement with the results of our past analyses of a number of HIV-1 and HIV-2 strains. The replicative capacities of the two sets of viruses were then measured in monocyte-derived macrophages. In a typical experiment, macrophages from several different donors were isolated by adherence to plastic, and their resting state was verified by measuring ^{3}H-thymidine incorporation two to three weeks later. At that point, the cells were infected with increasing amounts of singly-mutated Vpr-defective HIV-1 derivatives, or ones carrying mutations in Vpr and in either the MA NLS or the MA C-terminal tyrosine, and viral growth was monitored by measuring p24 antigen production in the supernatant. Several points could be made. First, no significant difference was noted between our and Freed et al.'s viruses. Second, the phenotype of the Vpr/MA tyrosine mutant viruses systematically correlated with that of the Vpr/MA NLS variants. Third, the phenotype of both types of MA/Vpr defective strains was linked to the multiplicity of infection (m.o.i.), so that these viruses exhibited wild-type replication kinetics when a high initial inoculum (i.e. 40 ng p24 antigen per $2x10^5$ cells) was used, whereas their growth was severely impaired when the cells were exposed to a lower dose of virus (i.e. 0.2 or 2 ng p24 antigen per $2x10^5$ cells, within the range of that used in all of our previous analyses). The m.o.i.-dependence of the phenotype of MA/Vpr double mutants in terminally differentiated macrophages, which will be fully described elsewhere, corroborates our observation that at least one additional NLS-bearing viral protein can function as a mediator of HIV-1 nuclear import, the influence of which is manifested both in vitro and in vivo (Gallay et al., 1996; Naldini et al. 1996; Gallay et al., submitted).

In summary, these results are consistent with the claim (Gallay et al., 1995a, 1995b; Bukrinskaya et al., 1996) that MA tyrosine phosphorylation participates in facilitating HIV-1 nuclear import. Even though under

some experimental conditions the point made by Freed et al. is valid, we feel at this stage that technical differences, for instance related to the use of different m.o.i.'s, explain the discrepancy between their and our results. Additional studies examining the composition and the processing of the HIV-1 preintegration complex should put the issue to rest.

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